IN THE SPECIFICATION

Please replace the specification with the substitute specification submitted herewith in marked-up and clean forms. Applicants hereby state that the substitute specification filed herewith contains no new matter.

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VECTOR FOR THE TRANSFORMATION OF *Phaffia rhodozyma* AND PROCESS OF TRASFORMATION THEREBY

FIELD OF THE INVENTION

The present invention is directed to a novel vector for transforming yeast and to a process for transforming yeast thereby. Particularly, the present invention is directed to a gene encoding L41, a ribosomal protein derived from *Phaffia rhodozyma* which is useful for producing natural pigment astaxanthin; a gene encoding a mutated L41 proteinhaving a cycloheximide-resistant activity; a ribosomal DNA derived from *Phaffia rhodozyma*; a vector for transforming *Phaffia rhodozyma* stably, comprising said gene encoding a mutated L41 protein and said ribosomal DNA; and a process for transforming *Phaffia rhodozyma* thereby.

BACKGROUND

Phaffia rhodozyma is a reddish yeast species producing astaxanthin, a useful natural pigment. Astaxanthin is a member of the carotenoids, which are represented by β-carotene, a precursor of vitamin A. Astaxanthin as a main pigment of curstacea, trout and salmon is widely distributed in nature. However, they cannot synthesize astaxanthin and should be supplied with it from a diet. Thus, it has been considered necessary to add the pigment in the cultivation of crustacea, trout and salmon, because the added pigments to the crustacea and fishes may attract the consumers and give better flavors to them. This carotenoid

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pigment plays key roles in the physiological metabolism of human as well as animals, with known effects suchan enhancement of immunological function, an antioxidant activity, a prevention of cancer and senescence, etc.

Because of increasing interests in *Phaffia rhodozyma* and pigments produced thereby, there have been a number of reports concerning a culture of *Phaffia rhodozyma*. However, these reports have been focused on how the inexpensive materials can be used for its culture, and have resulted in the development of method for culturing *Phaffica rhodozyma*, in which various local products may be employed, such as alfalfa juice (Okagbue *et al.*, *Appl. Microbiol. Biotechnol.*, 20, 33, 1984), molasses (Haard *et al.*, *Biotechnol. Lett.*, 10, 609, 1988), the byproducts of grape juice processing (Lango *et al.*, *Biotech. Forum Europe*, 9, 565, 1992), peat hydrolyzate (Martin *et al.*, 58, 223, 1993), the byproducts of corn wet-milling (Hayman *et al.*, *J. Ind. Microbiol.*, 14, 389, 1995), and the mixture of sugar cane extract, urea and phosphoric acid (Fontana, *et al.*, *Appl. Biochem. Biotechnol.*, 57/58, 413, 1996).

Although little is known about the genetics of *Phaffia rhodozyma*, the physiological features of *Phaffia rhodozyma* have been disclosed and the *Phaffia rhodozyma* mutant producing the pigment with high level has recently been selected (Johnson *et al.*, *Crit. Rev. Biotechnol.*, 11, 297, 1991; An *et al.*, *Appl. Environ. Microbiol.*, 55, 116, 1989; Chumpolkulwong *et al.*, *J. Ferment. Bioeng.*, 75, 375, 1997; Lewis *et al.*, *Appl. Environ. Microbiol.*, 56, 2944, 1990). In addition, a

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genetic analysis enlightened the ploidy and sexual cycle of *Phaffia rhodozyma*. In a flow cytometry study, Calo-Mata and Johnson found that no strain was haploid and that most were polyploid (Calo-Mata *et al.*, *Yeast Gen. Mol. Biol. Meet.*, 126, 1996). A pedogamic sexual process of conjugation has been also desclosed (Golubev *et al.*, *Yeast*, 11, 101, 1995).

Although *Phaffia rhodozyma* is potentially useful for the production of astaxanthin and the like, the pigment level in the wild type of *Phaffia rhodozyma* is very low. Therefore, there have been attempts to develop a novel mutant strain of *Phaffia rhodozyma*, which can produce the pigment more than usual one. However, these attempts have been hampered by the reduced growth rate and genetic instability of said mutant, which may occur when the pigment content in the mutant exceeds over the optimal range.

Another obstacle to the progress of the mutant is the method for mutagenesis. Chemical mutagenesis procedures have been performed conventionally, but it is associated with the simultaneous mutation of undesired genes leading to pleiotropic effects such as the reduction of growth rate, the prolonged induction time in the fermentation, etc. Furthermore, because the genome of the mutant strain is not stable, its subculture often decreases the yield of the pigment.

To solve these problems in the conventional breeding procedures and to enlarge the applicability of *Phaffia rhodozyma*, molecular breeding approaches have been initiated recently, using genetic transformation.

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However, since most of *Phaffia rhodozyma* strains are polyploid and thus cannot be made to be an auxotrophic variant by the method conventionally applied to yeast, it is preferable to employ an approach using antibiotics-resistant genes as selectable markers. More recently, there was reported a transformation system in which *Phaffia rhodozyma* actin promoter and G418-resistant gene were used for the transformation of *Phaffia rhodozyma*, However, the system showed poor transformation efficiency (Wery et al., Gene, 184, 89, 1997).

On the other hand, cycloheximide, an eukaryote-specific antibiotics, is applicable to the selection of yeast transformants. The target molecule of cycloheximide is ribosome and its target site is aminoacyl-tRNA binding site (A site) of ribosome, wherein it blocks peptidyl transferase activity of ribosome. As a result, it inhibits protein synthesis and cell growth in eukaryotes, without an effect on the organelles such as chloroplasts and mitochondria. Furthermore, it has been found that cycloheximide interacts with ribosomal protein L41, and that a mutation in L41 gene confers cycloheximide-resistance on the yeast transformants. Thus, cycloheximide and related mutant form of L41 gene are widely applicable to the process for transformation of yeasts.

Recent studies support the applicability of L41 gene to selectable marker in yeasts. Takagi et al. found that amino acid substitution through the mutagenesis of *Saccharomyces cerevisiae* L41 gene

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conferred cycloheximide-resistance, suggesting the usefulness of L41 gene as a selectable marker (Takagi *et al.*, *J. Bacteriol.*, 174, 254-262, 1992). Mutoh et al. proposed a biotechnological tool using *Candida maltosa* L41 gene as a selectable marker (Mutoh et al., *J. Bacteriol.*, 5383, 177, 1995). As it is well known that a substitution of 56th amino acid residue in the L41 protein conffer cycloheximide-resistant on Candida utilis (Keiji Kondo *et al.*, *J. Bacteriol.*, 7171, 177, 1995), transformation system using the substitution has been developed. Similar approaches have been introduced in *Kluyveromyces lactis* and *Schwanniomyces occidentalis* (Dehoux *et al.*, *Eur. J. Biochem.*, 213, 841-843, 1993; Pozo *et al.*, *Eur. J. Biochem.*, 213, 849-857, 1993). On algae *Tetrahymena*, the resistance is conferred by substitution of 40th amino acid residue, methionine to glutamine (Roberts *et al.*, *Exp. Cell. Res.*, 312, 81, 1973).

To overcome the foregoing and other disadvantages, we, the inventors of the present invention, have noted that cycloheximide and related mutation in L41 gene may be used to develop an efficient transformation system, whereby a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and the transformants are undoubtedly selected. To develop such system, we have constructed transforming vectors comprising the antibiotics-resistant gene and the targeting gene, which is used for the stable integration of foreign gene. We transformed *Phaffia rhodozyma* with asid vectors, according to a modified method for

electrotransforming *Cryptococcus neoformans*, a member of Basidiomycetes, whereto *Phaffia rhodozyma* belongs(Kim *et al.*, *Appl. Environ. Microbiol.*, 64, 1947, 1998).

The present invention is performed by cloning and sequencing *Phaffia rhodozyma* L41 gene; modifying the L41 gene by the mutagenesis of the region responsible to cycloheximide-resistance; constructing the vectors for transforming by inserting ribosomal DNA into the modified L41 gene; transforming *Phaffia rhodozyma* with the vector by electroporation method; and verifying the stable integration of the vector into the genome of the transformants.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a vector for transforming *Phaffia rhodozyma* efficiently.

It is a further object of the present invention to provide a recombinant vector for transforming *Phaffia rhodozyma*, which comprises the L41 protein of *Phaffia rhodozyma*.

It is an additional object of this invention to provide a L41 gene encoding the L41 protein of *Phaffia rhodozyma*, which has an antibiotics-resistant activity.

It is another object of this invention to provide a gene encoding a mutated L41 protein which can be used as a cycloheximide-resistant gene.

It is still another object of the present invention to provide a ribosomal DNA of *Phaffia rhodozyma*, which can be used to enhance the integration

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efficiency of foreign DNA into *Phaffia rhodozyma* genomes.

It is another object of the present invention to provide a process for transforming *Phaffia rhodozyma* by electroporation.

Further objects and advantages of the present invention will appear hereinafter.

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides an L41 gene encoding a ribosomal protein derived from *Phaffia rhodozyma*.

In addition, this invention provides a gene encoding mutated L41 proteinwherein the amino acid at the position 56 is replaced by glutamine. Since the amino acid residue is responsible for the cycloheximide-resistance, this mutated gene in a vector is useful for a selectable marker.

The present invention also provides a ribosomal DNA derived from *Phaffia rhodozyma*.

In addition, the present invention provides a recombinant vector comprising a gene encoding a protein having a cycloheximide-resistant activity and a ribosomal DNA derived from *Phaffia rhodozyma*.

In such aspect of the present invention, also provided is a recombinant vector, pTPLR1 comprising a gene encoding the muated L41 protein of *Phaffia rhodozyma* and a portion of the *Phaffia rhodozyma* ribosomal DNA.

The present invention also provides a process of

transforming *Phaffia rhodozyma* with the vector by electroporation.

In a preferred embodiment of the present invention, the vector is cleaved into a linear form. In another preferred embodiment of the present invention, the linearized vector is tintroduced into *Phaffia rhodozyma* using electrophoresis. In the more preferred embodiment of the present invention the electrophoresis is conducted with conditions as follows: electric pulse is 0.8~1.2 kV; an internal resistance is 400~800 Ω; and a capacitance is 25~50 μF.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is nucleotide sequence of genomic DNA containing upstream promoter region and coding region of a gene encoding L41 ribosomal protein of *Phaffia rhodozyma* (SEQ ID NO: 15) and deduced amino acid sequences by the gene (SEQ ID NO: 16), wherein,

Open boxes: TATA and CAAT sequences;

Underlined: the position of primers;

Bold letters: consensus sequence in splicing region of intron;

Open circle: amino acid residue at position 56

Figure 2 represents the construction of the pTPLR1 vector, its restriction map, a nucleotide sequcence for mutagenesis of L41 gene(SEQ ID NO: 17) and its amino acid sequence (SEQ ID NO: 18) and a mutated nucleotide

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sequence (SEQ ID NO: 19) and its amino acid sequence, wherein,

Numbers in parentheses: the sizes of inserts;

Blank boxes: DNA fragment containing L41 gene;

Grey boxes: rDNA fragments;

Black boxes: exons of L41 gene;

Thin lines: pBluescript SK(+) sequence;

Horizontal arrow: transcriptional direction of L41 gene;

X: *Xba*I site; S: *Sal*I site; C: *Cla*I site;

H: *Hind*III site; E: *Eco*RI site; Xh: *Xho*I site;

Sm: *Sma*I site; Bg: *Bgl*I site; Ba: *Bal*I site;

Kp: *Kpn*I site;

Figure 3 represents the restriction map of pTPLR1, the vector of the present invention,

Figure 4 represents the relationship between the condition of electroporation and the transformation efficiency or cell viability;

Figure 5 represents Southern blot analysis of pTPLR1 transformants, wherein,

C: nontransformant control;

1 to 5: pTPLR1 transformants;

Figure 6 is a schematicdiagram showing the mode of pTPLR1 integrated into the chromosome.

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DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is based upon the notion that cycloheximide and related mutation in a gene encoding L41 protein may be used to develop a transformation system, whereby a foreign gene is stably integrated into the genome of *Phaffia rhodozyma*, and the transformants are undoubtedly selected.

Hereinafter, the present invention is described in detail.

In one aspect, the present invention provides a gene encoding a L41 *Phaffia* ribosomal protein.

In a preferred embodiment, the genomic and cDNA sequences containing a gene encoding a L41 *Phaffia rhodozyma* ribosomal protein are prepared from a *Phaffia rhodozyma* strain (ATTC 24230).

The gene encoding L41 protein identified in the present invention shows high homology with other known L41 gene derived from yeasts, but contains 6 introns which have specific sequences in 5' and 3' regions of each intron. The genomic sequence represented by SEQ ID NO: 1 contains 7 exons and 6 introns and the cDNA encoding the L41 protein has a nucleotide sequence of 1,223 bp fragment represented by SEQ ID NO: 2. The deduced amino acid sequence is SEQ ID NO: 3. The proline at position 56 is responsible for sensitivity to cycloheximide (see FIG 1).

In another preferred embodiment, the cloned gene encoding L41 protein is modified by site-directed mutagenesis, so that the mutated L41 protein has a

cycloheximide-resistant activity. Particularly, a mutagenesis is performed to substitute the proline residue with glutamine, at the position 56 (see FIG 2).

The mutagenesis in the present invention includes all the possible modification of triplet codon in the amino acid position 56. For example, the codons for proline 56 may be replaced by all possible triplet codons for glutamine.

The present invention also provides a ribosomal DNA (hereinafter "rDNA") derived from *Paffia* yeast.

In this invention, rDNA means not only a DNA sequence which is transcribed to bear all types of eukaryotic ribosomal RNA, but also a non-transcription spacer (hereinafter, "NTS"), or a DNA sequence between the transcribed rDNA. rDNA can be preferably used to enhance the integration efficiency of foreign DNA into host genomes because rDNA sequence is highly repeated as tandem units in the eukaryotic genomes.

In a preferred embodiment, the rDNA is represented by SEQ ID NO: 4. The rDNA sequence contains NTS.

The present invention provides a recombinant vector for transforming Phaffia rhodozyma, comprising a cycloheximide-resistant gene and a rDNA.

According to one preferred embodiment, the cycloheximide-resistant gene is a gene coding a mutated L41 protein derived *Phaffia rhodozyma*.

According to one preferred embodiment, the rDNA may be used to enhance the integration efficiency of foreign DNA into the host genome.

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According to more preferred embodiment, the rDNA has a sequence of SEQ ID NO: 4.

According to another preferred embodiment, the gene encoding L41 protein of *Phaffia rhodozyma* is modified so as to have a cycloheximide-resistant acitivity and be used as a selectable marker in the recombinant vector (see fIG 2). This recombinant vector is useful for a stable transformation of a foreign gene into a host genome.

More particularly, the present invention provides pTPLR1, a vector for transforming yeasts. In the most preferred embodiment the recombinant vector comprises an NTS portion of *Phaffia rhodozyma* rDNA and a gene encoding a mutated L41 protein of *Phaffia rhodozyma* wherein the codon for proline at amino acid position 56 is substituted with the codon for glutamine (see FIG 3).

The recombinant vector of the present invention may be readily modified and improved within the spirits and scope of the present invention. For example, the recombinantvector of the present invention may include diverse L41 genes modified using various mutagenesis procedures and diverse rDNA sequences derived from various organisms.

In another aspect of the present invention, also provided is a process for transforming yeasts with foreign DNA. The process is based upon the established method for transforming *Cryptococcus neoformans*, but optimized to yeasts, using an antibiotics-resistance gene derived from yeasts instead of the bacterium-

derived counterpart.

In a preferred embodiment, the recombinant vector is cleaved into a linear form before transformation. The restriction enzymes used and the reaction may be selected carefully so that the foreign DNA is efficiently introduced into a host genome and only desired sequences of the vector are inserted to the host genome.

In the process for transforming of the present invention, an electroporation procedure is employed. According to another embodiment, electroporation is conducted with conditions as follows:an electric pulse of 0.8~1.2 kV, an internal resistance of 400~800 Ω, and a capacitance of 25~50 μF. After electroporation, the yeast cells are cultivated at 23°C for 14~16 hours, then spread on solid medium containing cycloheximide, and further cultivated at 23°C for 4~5 days. Assessing the effects of various conditions for the electroporation on the cell viability and the transforming efficiency (see FIG 4) reveals that abundant transformants are produced under such condition as electric pulse of 0.8 kV, an internal resistance of 600 Ω, and a capacitance of 50 μF.

In another embodiment, Southern blot analysis is used to verify the stable integration of foreign DNA (see FIG 5 and 6). The result confirms that the introduced genes are stably maintained in host genome, even after multiple subcultures on the medium without cycloheximide.

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EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The isolation of gene encoding a L41 protein of *Phaffia rhodozyma*

To isolate genomic DNA sequence encoding *Phaffia rhodozyma* ribosomal protein L41, we synthesized two PCR (polymerase chain reaction) primers, the sequences thereof were deduced from the nucleotide sequence of other yeast L41 genes and represented by SEQ ID NO: 5 (CYH1) and SEQ ID NO: 6 (CYH3). PCR was performed using the synthetic oligonucleotides, CYH1 and CYH3 as PCR primers and genomic DNA isolated from *Phaffia rhodozyma* (ATCC 24230) as template. A DNA fragment of 700 bp containing a gene encoding L41 protein was produced as a result, andthen was brought to a labeling reaction using digoxigenin (DIG)-labeling kit (Boehringer Mannheim, Germany) so as to be used as a probe for Southern blot analysis. To clone full-length genomic DNA encoding L41 protein, Southern blot hybridization was performed as desclosed by Sambrook et al. (Sambrook et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989) in a solution containing 5X SSC, 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, 5% blocking agent, and 50% (v/v) formamide,

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at 42°C. A strong hybridization signal was observed from an 8-kb *Xba*I fragment, and the *Xba*I fragments of 7 to 9-kb were isolated and ligated into pBluescript SK(+) (Stratagene, USA) to make a minilibrary. A clone (pTPL2), hybridizing with the PCR product was identified in a further Southern blot analysis wherein the DNA fragment of the minilibrary were blotted onto the membrane.

To identify the gene encoding L41 protein without intron, *Phaffia rhodozyma* L41 cDNA was isolated by the method of rapid amplification of cDNA ends (RACE) with 3'-RACE (GIBCO BRL, USA) and 5'-RACE (Clontech, USA) kits. Total RNA was prepared by the method of Elion and Warner (Elion *et al.*, *Cell*, 39, 663-673, 1984). Then mRNA was selected from the total RNA, using mRNA isolation kit (Novagen), and a 3' RACE reaction was performed with a synthetic oligonucleotide represented by SEQ ID NO: 7 as a 3' RACE primer, and 5' RACE reaction was ferformed with a synthetic oligonucleotide represented by SEQ ID NO: 8 as a 5' RACE primer.

The sequencing of the 3' and 5' RACE products suggested that a putative open reading frame of 1,223 bp be interrupted by six introns. The cloned gene encoding L41 protein was found to show high homology with those of other yeasts. However, the number of introns and their organization in the gene encoding the L41 protein of *Phaffia rhodozyma*were quite different from those of the other yeast. In fact, they have only one intron. GTPuNGT sequence and PyAG sequence were conserved in 5' and 3' ends, respectively, of the gene encoding L41 protein of *Phaffia rhodozyma;* this

conserved sequences were also reported in the intron of actin of *Phaffia rhodozyma*. The L41 gene encodes ribosomal protein comprising 106 amino acids, and most notably, proline at position 56 is identified to the amino acid residue responsible for the sensitivity to cycloheximide. The genomic DNA sequence of the gene encoding L41 protein of *Phaffia rhodozyma* was registered in GenBank on May 19, 1997, with accession NO. AF 004672 (see FIG 1 and SEQ ID NO: 15).

Example 2: a gene encoding mutant L41 protein having cycloheximide-resistant activity

To confer the cycloheximide-resistance on the genen encoding the L41 protein, we performed a site-directed mutagenesis which resulted in the amino acid substitution proline at position 56 with glutamine. Particularly, mutagenesis was carried out with the QuickChange in vitro mutagenesis kit (Stratagene) as described in the manufacturer's instructions with complementary mutagenic primers corresponding to amino acids 52 to 59 represented by SEQ ID NO: 9 and 10. The 2.2-kb *Sal*I fragment digested from the 8.0-kb fragment in Example 1 was replaced with the mutated fragment.

Example 3: The isolation of ribosomal DNA

Ribosomal DNA (rDNA) in the present invention was exploited to enhance the integration efficiency of foreign DNA into *Phaffia rhodozyma* genomes. To clone the rDNA fragment, two pairs of PCR primers, represented by SEQ ID NO: 11, 12 (corresponding to 18S

rDNA part) and 13, 14 (corresponding to 28S rDNA part), were designed from the known partial rDNA sequence of *Phaffia rhodozyma*.

By PCR with these two pairs of primers, two DNA fragments were obtained. One of those was 1.5-kb fragment containing the 5.8S rDNA NTS (non-transcription spacer) region with the primers represented by SEQ ID NO: 11 and 14, and the other was 6-kb fragment containing the 5S rDNA NTS region with the primers represented by SEQ ID NO: 12 and 13.

Two DNA fragments were used as a probe for cloning the rDNA unit in genomic Southern blot analysis, followed by the construction of minilibrary, as described in Example 1. Multiple rounds of Southern blot hybridization identified an 8.5-kb *Hin*dIII fragment, which was cloned and identity thereof was confirmed by partial sequencing. A 730-bp *Xho*I and *Xba*I fragment of the 8.5-kb fragment, which spans NTS region between 5S and 18S rDNA, was subcloned in pBluescript and the resulting vector was designated as pTPR4. A sequencing of pTPR4 enlightened that the cloned rDNA fragment showed high homology with 5.8S and 25S rDNA region of *Candida neoformans*, a member of Basidiomycetous yeasts including *Phaffia rhodozyma*. The 730-bp nucleotide sequence of *Phaffia rhodozyma* rDNA gene was registered in GenBank on July 28, 1997, with accession NO. AF 016256.

Example 4: The construction of recombinant vector for transforming *Phaffia rhodozyma*

To construct recombinant vectors for transforming

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Phaffia rhodozyma efficiently, we constructed pTPL5 vector containing the gene encoding mutated L41 protein prepared in Example 2 and pTPR4 vector containing the rDNA fragment prepared in Example 3 (see FIG 2). Particularly, pTPLR1 which is a recombinant vector for transforming *Phaffia rhodozyma* was cunstructed using the 3.7-kb fragment of pTPL5 as a cycloheximide-resistant marker and the 730-bp rDNA fragment of pTPR4 as a targeting sequence whereby a forein DNA is integrated into *Phaffia rhodozyma* genome with multicopy. The 3.7-kb *Xba*I-*Sal*I fragment of pTPL5 containing the gene encoding a mutated L41 protein was treated with the Klenow enzyme and inserted into the *Bal*I site of pTPR4. The resulting plasmid, pTPLR1 (see FIG 3), was introduced into *E. coli* DH5α strain, and the transformed *E. coli* strain was deposited in Korean Collection for Type Cultures (KCTC) on October 21, 1998 (accession NO: KCTC 0535BP).

We also constructed a plasmid, pTPLR2, which has the reverse direction agaist the coding sequences. The pTPLR1 and pTPLR2 vectors were digested with *Sma*I or *Bgl*I-*Kpn*I restriction enzymes, before the vector was brought to the transformation and integrated into the rDNA region of *Phaffia rhodozyma* genome.

Example 5: The transformation of *Phaffia rhodozyma* with pTPLR1 vector

To transform *Phaffia rhodozyma* with the pTPLR1 vector efficiently, we developed the transformation method, which is based upon the method for transforming a Basidiomycetous yeast, *Cryptococcus neoformans* (Varma

et al., Infect. Immun., 60, 1101, 1992) but optimized for *Phaffia rhodozyma*. Electroporation procedure was employed in the process of the present invention. Particularly, *Phaffia rhodozyma* cells from a log-phase cluture in 50 ml of YM medium were harvested by centrifuge at 3,000 rpm for 10 minutes, then washed twice with equal volume of electroporation buffer (270 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, pH 8.0) containing 1 mM dithiothreitol (DTT), and resuspended in the electroporation buffer without DTT. The linearized plasmid pTPLR1 (200 ng) was mixed with a 50 µl aliquot (approximately 2×10^7 cells) of the cell suspension, and transferred to a cuvette (0.2-cm electrode gap; Bio-Rad, USA). Sets of electroporation were performed(Gene Pulser II; Bio-Rad, USA) under the various ranges of electric pulse (0.8 to 1.2 kV), internal resistance (400 to 800 Ω) and capacitance (25 to 50 µF). The electroporated cells were resuspended in 1 ml of YM medium and transferred to a test tube for incubation. After being shaken for 12 to 16 hours at 23°C, cells were spread on YM agar medium containing 10 µg/ml of cycloheximide and incubated at 23°C for 4 to 5 days.

Figure 4 shows the relationship between the condition of electroporation and the transformation efficiency or cell viability. The transformation efficiency was mainly dependent on the capacitance, preferably of 50 µF rather than 25 µF. In summary, more transformants were produced when an electric pulse of 0.8 kV was delivered and internal resistance of 600 Ω was set with a capacitance of 50 µF, generating

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pulse lengths of 18 to 20 ms. Under such a condition, approximately 30% of cells survived, and transformation efficiencies of 800 to 1000 transformants per μg of DNA could be routinely obtained with pTPLR1 linearized either by *Sma*I or by *Bgl*II-*Kpn*I.

Using the optimized process, we transformed *Phaffia rhodozyma* with various vectors and observed the colony formation on the YM agar medium containing cycloheximide.

Interestingly, there was no transformant with pTPLR2 in any condition, suggesting that L41 gene is expressed only when the transcriptional direction of the integrated L41 gene is the same as that of rDNA.

Without the linearization of pTPLR1 before transformation, no colony was formed. This may result from the fact that rDNA does not have the autonomous replication sequence (ARS) or its similar function.

A vector carrying a gene encoding a mutated L41 protein having cycloheximide-resistant activity but not containing rDNA sequence, was introduced into *Phaffia rhodozyma*. In this case, a few colonies were observed. We suspected that the mutated L41 gene in the vector would replace endogenous L41 gene in the genome, rather than be integrated in directed position.

In addition, we transformed *Phaffia rhodozyma* with a vector wherein the promoter of L41 gene was deleted, and observed transformed colonies. The Southern blot analysis of this transformant showed the same hybridization pattern as that of nontransformant control. This indicates that the a substitution has

occurred in this case, rather than an integration in the directed position.

Example 7: Southern blot analysis of the transformants

To assess the stability of the introduced foreign DNA in *Phaffia rhodozyma* genome according to the present invention, a Southern blot analysis of genomic DNA, which is prepared from pTPLR1 transformants or nontransformant control was performed (see FIG 5). Particularly the genomic DNA was digested with *Sma*I or *Eco*RI enzyme, and the 2.2-kb *Sal*I fragment of pTPL2 was used as a probe in the hybridization. The intensity of colored band was measured by the scanning densitometer (Model GS-700 Imaging Densitometer, Bio-Rad, USA).

Southern blot analysis, wherein genomic DNA of transformants was digested with *Sma*I, showed two colored bands at 9.0-kb and 4.1-kb. A signal at 9.0-kb is observed both in a nontransformant control and in the transformants, indicating that this band originated form the endogenous gene encoding L41 protein of *Phaffia rhodozyma*. A much stronger signal at 4.1-kb also was detected in transformants, but not in the control. This was identical with the result of the restriction map of the transforming plasmid (see FIG 6). The size and relative intensity of signal at 4.1-kb suggested that multiple copies (approximately, 7 copies) of the transforming plasmid had been integrated.

In another Southern blot analysis with *Eco*RI digestion, two bands at 5.8-kb and 2.8-kb were found only in transformants (see FIG 5). The 5.8-kb band

originated from a 3.2-kb rDNA fragment and a 2.6-kb L41 gene fragment, and the 2.8-kb band originated from a 1.7-kb rDNA fragment and a 1.1-kb L41 gene fragment. Integration may occur as diagrammed in Figure 6.

These results were reproducible in Southern blot analysis with rDNA probe. Most importantly, copy number did not decrease after a prolonged cultivation in YM medium with or without cycloheximide, indicating that the transforming plasmid was integrated into the chromosome and maintained stably.

INDUSTRIAL APPLICABILITY

As shown above, the vector for transforming *Phaffia rhodozyma* of the present invention comprises rDNA and a gene encoding a mutated L41 protein having cycloheximide-resistant activity, which is useful for a stable integration of foreign DNA into host genome and for a convenient selection of transformants, respectively. The vector of the present invention is, therefore, applicable to the transformation of yeast cells including *Phaffia rhodozyma*, in combination with the process for transforming yeast cells of the present invention, wherein the yeast cells are transformed through the optimized electroporation.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for

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carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.